Podocyte-Derived BMP7 Is Critical for Nephron Development

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ABSTRACT

Individuals with congenital renal hypoplasia display a defect in the growth of nephrons during development. Many genes that affect the initial induction of nephrons have been identified, but little is known about the regulation of postinductive stages of kidney development. In the absence of the growth factor bone morphogenic protein 7 (BMP7), kidney development arrests after induction of a small number of nephrons. The role of BMP7 after induction, however, has not been fully investigated. Here, we generated a podocyte-specific conditional knockout of BMP7 (Bmp7fl/fl;Nphs2-Cre+ [BMP7 CKO]) to study the role of podocyte-derived BMP7 in nephron maturation. By postnatal day 4, 65% of BMP7 CKO mice had hypoplastic kidneys, but glomeruli demonstrated normal patterns of laminin and collagen IV subunit expression. Developing proximal tubules, however, were reduced in number and demonstrated impaired cellular proliferation. We examined signaling pathways downstream of BMP7; the level of cortical phosphorylated Smad1, 5, and 8 was unchanged in BMP CKO kidneys, but phosphorylated p38 mitogen-activated protein kinase was significantly decreased. In addition, β-catenin was reduced in BMP7 CKO kidneys, and its localization to intracellular vesicles suggested that it had been targeted for degradation. In summary, these results define a BMP7-mediated regulatory axis between glomeruli and proximal tubules during kidney development.

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then in the early tubules derived from the mesenchyme. As the nephron develops, BMP7 is expressed in podocytes of the maturing glomeruli. Because the size of each nephron increases during maturation of the kidney, we hypothesized that podocyte-derived BMP7 might be involved in driving tubular epithelial expansion. Thus, we generated a podocyte-specific conditional knockout mouse model of BMP7 (BMP7 CKO mice) and analyzed postnatal kidney development.

RESULTS

Localization of BMP7 and Its Receptors in Postnatal Kidneys

The major compartments of BMP7 expression are the derivatives of the ureteric bud, early nephrons, and the podocytes of immature glomeruli (Figure 1A). In situ hybridization localization of BMP receptors showed expression mainly in the nephrogenic zone of wild-type newborn kidneys (Figure 1B), consistent with previous reports, but also demonstrated wide expression pattern throughout the kidneys, including tubules and glomeruli.

Generation of Podocyte-Specific Conditional Knockout Mouse Model of BMP7

Mice with floxed BMP7 exon 1 (BMP7<sup>lox/lox</sup>) were crossed with mice that express the Cre-recombinase from the <i>NPHS2</i> gene promoter (see the Concise Methods section for the strain used in this study) (Supplemental Figure 3), finally obtaining mice homozygous for the BMP7 floxed allele and carrying the <i>NPHS2-Cre</i> transgene. During the course of the genotyping using tail DNA (Supplemental Figure 3), we observed that approximately one third of tail DNA showed evidence of recombination between <i>LoxP</i> sites, even though examination of recombination in the kidney showed the Nphs2-Cre to be podocyte specific (Supplemental Figure 1). When present, the extent of recombination between <i>LoxP</i> sites was variable, suggesting it did not result from germ line recombination; however, the degree of recombination in tail DNA correlated with the most severely affected kidneys. This is consistent with the expectation that kidneys in which there was already one recombined allele would require only a single, instead of two, Nphps2-Cre-mediated recombination events to inactivate BMP7 fully in podocytes and would show more dramatic phenotypes. Because the recombination at <i>LoxP</i> sites did not seem to be an inherited event, the BMP7<sup>lox/lox</sup> genotype designation is useful in this report regardless of whether some degree of Cre-mediated recombination was observed in tail DNA. Importantly, true BMP7 null heterozygotes are reported to have normal kidney development, such that the phenotype reported here is indeed due to the loss of BMP7 expression by podocytes and is not a BMP7 heterozygous phenotype. Littersmates that were homozygous for the floxed BMP7 allele but were negative for NPHS2-Cre (BMP7<sup>lox/lox</sup>/Nphs2-Cre<sup>−/−</sup>) were used as controls. Of note, the BMP7 floxed allele used in

![Figure 1. Expression of BMP7 and its receptors in postnatal kidneys.](image-url)
this study still carries the neomycin resistance gene (neo<sup>1</sup>); however, comparison of BMP7<sub>lox/lox</sub> mice and wild-type littermates revealed no differences caused by the neo<sup>1</sup> allele by itself. Mice of all genotypes were born in the expected Mendelian frequency.

To evaluate the ability of the NPHS2-Cre recombinase to excise the floxed BMP7 alleles in a podocyte-specific manner in developing kidneys, we examined BMP7 mRNA expression by in situ hybridization in newborn and postnatal day 4 (P4) kidneys. In control kidneys, BMP7 expression was observed in podocytes of immature glomeruli (Figure 1A, a, c, and e) in addition to ureteric bud derivatives within the nephrogenic zone; however, in BMP7 CKO kidneys at P0 and P4, the expression was missing specifically in podocytes (Figure 1A), whereas ureteric bud expression was unchanged. A total of 142 (90%) of 158 glomeruli counted from five sections taken from P0 kidneys showed little or no BMP7 expression; among control glomeruli, 97% showed BMP7 expression.

**Hypoplasia of BMP7 CKO Kidneys**
At P0, both control and BMP7 CKO mice (n = 6 mutants) showed the typical gradient of renal development with normal-appearing nephrogenic zones (data not shown); however, by P4, approximately 65% (19 of 29) of BMP7 CKO mice had hypoplastic kidneys (Figure 2A, b versus a). Among all ages examined between P4 and P10, 40 of 72 BMP7 CKO mice had hypoplastic kidneys ranging from 70 to 20% normal size, compared with five of 77 control mice that had slightly hypoplastic kidneys. In BMP7 CKO kidneys, the nephrogenic zone was largely missing by P4 with only a single layer of maturing nephrons in the most dramatic cases, and the kidneys had irregular surface (Figure 2A, d versus c). Glomeruli appeared

![Figure 2](image_url)

Figure 2. Histologic analysis of kidneys. (A) P4 histology and P7 electron microscopy. Genotypes are designated at the top of each column. (a and b) Low-power views of BMP7<sub>lox/lox</sub> (a) and BMP7<sub>lox/lox</sub>;Nphs2-Cre<sup>−</sup> (b) mice. (c and d) Cortex of a and b. Bar = 0.5 mm in a; 100 μm in c. The nephrogenic zone (NZ) is demarcated. G, Mature glomeruli; g, immature glomeruli. Immature glomeruli are not recognizable in BMP7<sub>lox/lox</sub>;Nphs2-Cre<sup>−</sup> kidneys, and the NZ is absent along much of the periphery of the kidney. (e and f) High-power views of glomeruli. (g and h) Electron microscopy of P7 glomeruli; some foot process effacement is evident in BMP7<sub>lox/lox</sub>;Nphs2-Cre<sup>−</sup> (h). Bar = 33 μm in e; 2.5 μm in g. (B) Histology of P10 kidneys. (a and b) Low-power views. (c and d) Medium-power views of cortex. (e and f) High-power view of glomeruli. Designations, bars as in A.
normal in mutants at P4 (Figure 2A, f versus e), although, by P7, in the most affected kidneys, we found proteinaceous material in some tubules (data not shown) and observed mild podocyte foot process effacement (Figure 2A, h versus g).

**Impaired Nephrogenesis in BMP7 CKO Kidneys**

To examine further why fewer nephrons were present in BMP7 CKO kidneys, we examined markers of early nephrogenesis, including BMP7 itself. At P0, BMP7 is mainly found in ureteric bud derivatives and to a lesser extent in the cap mesenchyme (Figure 1Ac). By P4, the ureteric bud derivatives and cap mesenchyme are no longer present, and structures most compatible with comma- and S-shaped bodies are present in the most peripheral zone (Figure 1Ae). The expression of BMP7 seems diminished in S-shaped bodies of mutant kidneys at P4 (Figure 1Af), suggesting that BMP7 expression in the presumptive podocytes is being lost and that this has a marked effect on nephrogenesis.

Consistent with this histology, Wnt418 was also expressed in comma- and S-shaped bodies at P4 in control kidneys (Figure 3C), but analogous structures generally appeared less well developed in BMP7 CKO kidneys (Figure 3D). Wt1, as a marker of glomeruli,3 also seemed to be expressed abnormally in mutant kidneys (Figure 3, E and F), with fewer capillary loop glomeruli present, replaced by abnormal Wt1-expressing structures that probably represented dysplastic glomeruli. Finally, staining with Lotus Tetragonolobus (TG) lectin, to define proximal tubule structure, demonstrated more poorly differentiated structures in the subnephrogenic zone area in BMP7 CKO kidneys as compared with control (Figure 3, G and H). These results indicate a role for BMP7 in the postinductive stage of nephrogenesis; however, at a later stage of development, at P10, mutant kidneys remained hypoplastic, but the histology was relatively normal, suggesting that dysplastic nephrons either achieved some degree of maturation or were not retained after nephrogenesis was complete (Figure 2B).

**Glomerular Maturation in BMP7 CKO Kidneys**

Although there was a defect in nephron development in BMP7 CKO kidneys, the initial round of nephrons appeared to be structured normally. Glomeruli of BMP7 CKO kidneys seemed to have undergone the usual conversion in laminin and type IV collagen isoforms19 and other markers of podocyte and glomerular differentiation such as podocin, α3β1 integrin, platelet-endothelial cell adhesion molecule (CD31), and desmin were expressed normally in BMP7 CKO glomeruli (Figure 4).

**Reduced Nephron Size in BMP7 CKO Kidneys**

Cebrian et al.7 quantitatively demonstrated in murine kidneys that the number of nephrons increases dramatically from the late embryonic to early postnatal period, in parallel with the growth of a kidney. Because a proximal tubule comprises the largest portion of a single nephron’s mass,20 its size should have a significant impact on the size of each nephron as well as the overall size of the kidney. Decreased number of TG lectin–stained proximal tubular sections per WT1-stained glomeruli in BMP7 CKO kidneys demonstrated reduced proximal tubular mass (Figure 5) and, thus, indicated reduction of nephron size.

**Decreased Cellular Proliferation of Proximal Tubules in BMP7 CKO Kidneys**

Immunohistochemistry to Ki-67, which is a marker for cellular proliferation, demonstrated a significant decrease in Ki-67–positive cells within proximal tubules of mutant compared with control kidneys (Figure 6, A through E). No difference in Ki-67 staining within glomeruli of BMP7 CKO kidneys was observed (Figure 6F), indicating that decreased cellular proliferation was specific to proximal tubules. Terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling TUNEL staining did not reveal a significant
difference in apoptosis between mutant and control kidneys (data not shown).

Canonical Smad Pathway Was not Involved in the BMP7 Effect on Nephron Growth

Because Smad1, 5, and 8 are known to transduce signals upon interaction of BMP7 with its receptors,21–23 we examined the phosphorylation of Smad1, 5, and 8 (Figure 7). Immunohistochemistry demonstrated wide distribution of phosphorylated Smad1-, 5-, and 8-positive (phospho-Smad1, 5, and 8) cells in both control and BMP7 CKO kidneys (Figure 7, A and B). No significant difference in numbers of phospho-Smad1, 5, and 8 cells were observed between control and mutant proximal tubules, however; neither did Western blot of tissue from isolated cortex, from which the medullary papilla had been removed, reveal a difference in the level of phospho-Smad1, 5, and 8 (Figure 7C).

Increased Inhibitory Smad7 in BMP7 CKO Kidneys

Smad7 has been identified as a member of the inhibitory Smads of the TGF-β superfamily signaling pathways.24 Smad7 may also be involved in noncanonical BMP signaling, as it has been shown to interact with p38 mitogen-activated protein kinase (MAPK) and β-catenin.25,26 To explore the presence of negative regulatory system for canonical Smad pathway, we examined expression of Smad7 by immunohistochemistry (Figure 8). In control kidneys, we noted Smad7 expression in nephrogenic zone, glomeruli, and tubulointerstitial region (Figure 8, A and C), consistent with the findings of previous reports.27,28 In BMP7 CKO kidneys, cytoplasmic expression of Smad7 was increased specifically in proximal tubular epithelium (Figure 8, B and D) that was identified by double-staining with TG lectin (Figure 8, E and F).

Involvement of Smad-Independent Pathways

BMP also activate Smad-independent intracellular signaling pathways.29 In this study, because BMP7 maintained nephron growth through cellular proliferation of proximal tubular epithelium (Figure 6), we investigated the involvement of other signaling pathways that are known to promote cellular proliferation during organogenesis, such as p38 MAPK pathway and β-catenin-mediated pathways.

Phosphorylated p38 MAPK Localization.

Recently, p38 MAPK was demonstrated to be activated by BMP7 and to promote cellular proliferation in cultured renal collecting duct cells.30 Immunohistochemistry demonstrated wide distribution of phosphorylated p38 MAPK (phospho-p38 MAPK)-positive cells throughout the control kidneys (Figure 9A); however, in BMP7 CKO kidneys, we observed decreased staining specifically in proximal tubules (Figure 9, B and D). Western blot analysis of tissue from isolated cortex demonstrated marked decrease of phospho-p38 MAPK in BMP7 CKO kidneys (Figure 9E), consistent with the immunohistochemistry (Figure 9, B and D, compare with C and E).

β-Catenin Localization.

β-Catenin, a major downstream effector of “canonical” Wnt signals, is known to be involved in regulating the proliferation of epithelial cells during organogenesis.31 Although Wnt signals that specifically affect proximal tubular proliferation are yet to be described, it is also known that BMP signals may affect β-catenin expression during organogenesis.32 In control kidneys, immunofluorescence staining
demonstrated basolateral expression in proximal tubules (Figure 10, A and C). In marked contrast, in BMP7 CKO kidneys, β-catenin was highly concentrated within intracellular vesicles of proximal tubular epithelium, suggesting it had been targeted for degradation (Figure 10, B and D). Western blots demonstrated a decreased amount of intact β-catenin in the cortex of BMP7 CKO kidneys (Figure 10E), with a shifted band suggestive of increased phosphorylation, consistent with its being targeted for degradation.

**DISCUSSION**

This study was designed to assess the role of podocyte-derived BMP7 in nephron maturation. The original BMP7 knockout mice, developed independently by Dudley et al. and by Luo et al., demonstrated an essential role for BMP7 in kidney development. There was a premature loss of the progenitor population, and severely hypoplastic kidneys that led to neonatal lethality were produced. In this study, the use of a conditional allele of BMP7 has allowed us to distinguish the role of BMP7 expressed from specific cell lineages, in this case podocytes, and has allowed the study of postnatal phenotypes that focus more specifically on nephron development. Here we show that podocyte expression of BMP7 has an important role both in early nephron development and in nephron maturation. BMP7 seems to signal through Smad-dependent and -independent pathways and regulate localization of β-catenin in developing tubules.
In the absence of podocyte expression of BMP7, nephron development seemed abnormal. In the most dramatically affected conditional mutant mice, many nephrons induced after the first round were severely dysgenic. This is reminiscent of the original BMP7 mutant mice, although the conditionally mutant mice developed to a much greater extent than the original null mice. Several explanations—that are not mutually exclusive—can be suggested to account for the difference between the podocyte-specific knockout and the original BMP7 null mice. First, the ureteric bud derivatives and their derivative collecting ducts also express BMP7 (Figure 1A). Consistent with this expression pattern, the development of the collecting system proceeds relatively normally in the conditional mutant mice in contrast to the null mice (Figure 2, A and B). Second, the distinct differences in the extent of development between the earliest nephrons and subsequent rounds suggest either that the earliest nephrons are less dependent on BMP7 in general or that they are less dependent on podocyte-derived BMP7. Because there are not great morphologic differences between early and late nephrons (with the exception of the extension of the loop of Henle into the medulla), and, to date, no known molecular genetic differences are known in the regulation of their development, it seems unlikely that there is a differing overall requirement for BMP7 among early and late nephron populations. Instead, it is possible that the earliest nephrons have access to other sources of BMP7, possibly from the ureteric bud derivatives, cap mesenchyme, or possibly maternal sources. As the nephrogenic zone develops into a more distinct structure, BMP7 expression in the cortex becomes progressively more restricted to ureteric bud derivatives and podocytes (Figure 1Aa). By P4, ureteric bud derivatives have largely disappeared, and podocytes remain the major location of BMP7 expression in the developing cortex (Figure 1Ac). Thus, it is likely that nephron development becomes progressively more dependent on podocyte expression of BMP7 during the course of nephrogenesis. It is also possible that the conditional mutation of the BMP7 locus was more efficient in later rounds of nephrons, although this is not suggested by our in situ analysis of BMP7 expression in conditional mutant mice.

As previously discussed, BMP proteins signal both through Smad-dependent and -independent pathways. For example, signaling through p38 MAPK seems to be im-

Figure 7. Phospho-Smad1, 5, and 8 proteins expression. (A and B) Immunohistochemistry using antibody specific for phospho-Smad1, 5, and 8 (brown) in the cortex region of P4 kidneys. (A) BMP7lox/lox mice. (B) BMP7lox/lox;Nphs2-Cre mice. Bar = 100 μm. (C) Western blots of kidneys from BMP7lox/lox and BMP7lox/lox;Nphs2-Cre mice. The antibody used in the blot is designated to the left of each panel. Seventy-five micrograms of protein extracted from the renal cortex of a single P10 kidney of an individual mouse was loaded in each lane. The amount of phospho-Smad1, 5, and 8 proteins was controlled by the quantity of total Smad1, 5, and 8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins.

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Figure 8. Smad7 protein expression in P4 kidneys. (A through D) Immunohistochemistry using antibody for Smad7 (brown). (A, C, and E) BMP7lox/lox;Nphs2-Cre mice. (B, D, and F) BMP7lox/lox;Nphs2-Cre mice. (A and B) Cortex. (C and D) Proximal tubules. Arrows indicate proximal tubules showing increased Smad7 in D and less staining in C. (E and F) Merged images with immunofluorescence staining for TG lectin (green) to indicate the location of proximal tubules. Bar = 100 μm in A; 50 μm in C and E.
important in the development of the collecting system; however, it remains to be determined whether there is a clear distinction between so-called Smad-dependent and -independent pathways. On the one hand, staining for phospho-Smad1, 5, and 8 was not different between control and mutant kidneys. On the other hand, abundant staining and detection of phospho-Smad1, 5, and 8 on Western blots indicated that Smad signaling was indeed in effect in proximal tubules. In addition, increased Smad7 staining in mutant proximal tubules, along with decreased p38 staining, suggested a possible interaction between MAPK signaling and Smad-dependent signals. Supporting this possibility are reports of physical interactions between Smad7 and p38 that may regulate the abundance of p38, thereby affecting proliferation. Smad7 has been shown to be induced by stimulation with TGF-β. Because Smad7 is an inhibitory Smad, it has been suggested to be part of a negative feedback loop to regulate TGF-β signaling. Our results suggest that in contrast to TGF-β signals, BMP7 signals suppress Smad7 expression. Although it remains unclear whether Smad1, 5, and 8 have a role upstream (or downstream) of Smad7 in driving epithelial proliferation, it is possible that the increased levels of Smad7 provide an indication that epithelial proliferation is regulated by a balance between signals downstream of BMPs and TGF-β.

BMP7 has shown promise as a therapeutic agent to preserve renal epithelia. This raises the question of whether there is normally ongoing expression of BMP7 in the adult kidney and, if so, what its source is. The podocyte expression of BMP7, as judged by in situ hybridization, seems to diminish in the most...
mature glomeruli; however it is possible that expression of BMP7 in podocytes continues at a low level throughout adulthood. If this is the case, then it raises the question of whether tubule damage, that may follow glomerular damage, is in part due to a loss of BMP7 expression by the glomerulus. Furthermore, it remains possible that the phenotype we observed is due to secondary effects and that BMP7 is acting completely within the glomerulus. Further studies examining the presence of BMP7 protein will be required to answer this question. In addition, it was previously published that ectopic glomerular expression of Noggin, a BMP antagonist, led to glomerular damage in older mice. The full range of BMP expression in the glomerulus is not known, so it cannot be determined whether the Noggin effect relates specifically to BMP7 function. Moreover, our studies have focused on developmental phenotypes, and further study of older mice will be required for full examination of the function of BMP7 in the glomerulus.

**CONCISE METHODS**

**Targeted Inactivation of BMP7 in Podocytes**

A new strain of NPHS2-Cre mice was derived in our laboratory using a construct obtained from Dr. Susan Quaggin (Mt. Sinai Hospital, Toronto, Ontario, Canada). Characterization of this strain is shown in Supplemental Figures 1 through 3. NPHS2-Cre mice were crossed with BMP7flox/flox, which contain loxP sites upstream and downstream of exon 1 of the BMP7 gene, in two successive rounds of breeding to obtain BMP7flox/flox;Nphs2-Cre+ mice, referred to as BMP7 CKO mice. A detailed report on the construction of this targeting vector and derivation of mutant mice has been submitted (D.G. and A.N.E., in preparation). All procedures with animals were approved by institutional animal care and use committees.

**Genotyping**

Genotyping information is presented in Supplemental Figure 3.

**In Situ Hybridization**

Tissue in situ hybridization was performed as described previously. Riboprobes were obtained or generated from the coding region of mouse: BMP7 (obtained from J.M. Wozney; Genetics Institute, Cambridge, MA), ALK3,43 ALK6,44 ActRII (obtained from V. Rosen, Harvard School of Dental Medicine, Boston, MA), BMPRII (obtained from L. Gamer; Harvard School of Dental Medicine, Boston, MA), Wnt4,18 and WT15 (generated in our laboratory). Sense and antisense probes were synthesized and labeled with digoxigenin-UTP (Roche, Mannheim, Germany).

**Immunofluorescence Staining**

Immunofluorescence staining was performed as described previously. Primary antibodies used were as follows: Mouse anti-WT1 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), TG lectin (1:100; Vector Laboratories, Burlingame, CA), and mouse anti–β-catenin (1:100; BD Transduction Laboratories, San Jose, CA). Rabbit anti-laminin α2, β1, β2, and γ1 and collagen IV α2 and α4 were used as described previously. Fluorescence images were captured off a Nikon Eclipse 800 microscope with a Spot 2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI) using Spot Software 2.1. Secondary antibody alone was consistently negative on all sections.

**Immunohistochemistry**

Five-micrometer paraffin sections of 4% paraformaldehyde-fixed kidneys were placed in citrate-buffered solution (pH 6.0) and then boiled for 30 min for antigen retrieval. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and nonspecific binding was blocked with 10% BSA. Primary antibodies used were as follows: Rabbit anti–Ki-67 (1:150; Vector Laboratories); anti–phospho-Smad1, 5, and 8 (1:100; Cell Signaling Technologies, Danvers, MA); anti–Smad7 (1:50; Santa Cruz Biotechnology); and anti–phospho-p38 MAPK (1:50; Cell Signaling Technologies). Diaminobenzidine substrate (Sigma Chemical Co., St. Louis, MO) was used for the color reaction. For Ki-67 staining, sections were counterstained with hematoxylin. Secondary antibody alone was consistently negative on all sections. Proximal tubules were distinguished from distal tubules in the cortex area by their luminal brush borders and relatively large cytoplasmic structures.

**Programmed Cell Death Analysis**

The analysis was carried out according to the manufacturer’s protocol, using Apoptag in situ apoptosis detection kit (Chemicon Int., Temecula, CA).

**Immunoblotting**

Immunoblotting was performed as described previously. Briefly, kidneys were dissected from mice at P10. After dividing the harvested kidneys into cortex and medulla, the cortex was homogenized in 800 μl of RIPA buffer for protein extraction. The following primary antibodies were used: Rabbit anti–phospho-Smad1, 5, and 8; anti–Smad1, 5, and 8 (Santa Cruz Biotechnology); anti–phospho-p38 MAPK and anti–p38 MAPK (Cell Signaling Technologies); mouse anti–β-catenin (BD Transduction Laboratories); and rabbit anti–glyceraldehyde-3-phosphate dehydrogenase (Invitrogen, Carlsbad, CA).

**Statistical Analysis**

All results are expressed as means ± SEM. Paired evaluations were made for the comparison between control and BMP7 CKO groups. Statistical significance between means was determined with t test. P < 0.001 was considered significant.

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